Br. J. exp. Path. (1987) **68**, 71–80

Antiserum-induced neutropenia in the rat: characterization of a rabbit anti-rat neutrophil serum

Håkan Sandler, Herman Högstorp, Claes Lundberg* and Bengt Gerdin*†
*Departments of Forensic Medicine and †Surgery, University of Uppsala and *Department of Experimental Medicine, Pharmacia AB, Uppsala, Sweden*

Received for publication 11 December 1985
Accepted for publication 16 September 1986

Summary. A heterologous rabbit anti-rat neutrophil serum (ANS) based on peptone-stimulated peritoneal exudate neutrophils (PMNLs) from Sprague Dawley rats was prepared. Leucoagglutination and indirect immunofluorescence assays revealed high titres of antibodies to rat PMNLs (1/2560), lower titre of antibodies to rat lymphocytes (1/160) and a very low titre against rat platelets (1/20). ANS given intravenously (i.v.) to rats in doses of up to 42 mg of protein/kg b.w. caused transient neutropenia, lasting about 10 min after administration, and thrombocytopenia, lasting about 5 min. Two minutes after an i.v. injection of 21 mg of ANS/kg b.w. there was profound uptake of ^{51}Cr-labelled PMNLs in the lung, increased release of ^{51}Cr to plasma, an increased amount of ^{51}Cr in the spleen and consumption of $>98\%$ of total complement (CH_{50}). Two hours later there was high activity of ^{51}Cr in the plasma, spleen and liver, while lung radioactivity had decreased to below baseline and CH_{50} had recovered to 55% of baseline. An intraperitoneal (i.p.) injection of ANS was followed by prolonged neutropenia with a maximum after 12 h. Simultaneously peripheral mononuclear cells slightly decreased. There was no change in the number of peripheral platelets in the blood or in the plasma concentration of fibrinogen, α_2-antiplasmin, plasminogen or plasminogen activators. Intraperitoneal administration of ANS did not affect CH_{50}. It was concluded that the raised ANS had good specificity against rat PMNLs and was able to induce prolonged neutropenia after i.p. injection without affecting the complement of fibrinolytic system.

Keywords: neutrophils, rats, neutropenia, antiserum

The polymorphonuclear leucocyte (PMN) is known to participate in the pathogenesis of a large number of conditions with inflammatory characteristics, e.g. in postischaemic blood flow disturbances and posttraumatic reactions in the lung. The involvement of PMNs is usually suspected when a local accumulation of these cells is observed in the organ in question. Firm conclusions as to the pathogenic role of PMNs, however, require evidence that the condition in question can be counteracted under experimental conditions by depleting the organism of its PMNs. Such studies have mostly employed different cytotoxic or antineoplastic agents, e.g. nitrogen mustard (Wedmore & Williams 1982;

Correspondence: Dr Håkan Sandler, Dept. of Experimental Medicine, Pharmacia AB, S-751 82 Uppsala, Sweden.

Shasby *et al.* 1982), cholchicine (Flick *et al.* 1981) and hydroxyurea (Johnson & Malik 1980; Tahamont & Malik 1983). These and similar drugs have a broad action on all dividing cells and this lack of selectivity makes it difficult to interpret the results obtained when drugs are used in different experimental conditions.

The most attractive way of eliminating PMNs from the organism would be to use a specific antiserum raised against PMNs from the species in question.

In some species there are acceptable schedules for obtaining neutropenia, e.g. administration of hydroxyurea in the sheep and dog (Johnson & Malik 1980; Tahamont & Malik 1983) and nitrogen mustard in the rabbit (Wedmore & Williams 1981), but in the rat it is virtually impossible to induce neutropenia by means of cytotoxic agents without serious side effects. Antineutrophil sera (ANS) have been utilized in several species in investigations on the dynamics of myelopoesis after systemic depletion of PMNs (Lawrence *et al.* 1967), in studies on the Arthus phenomenon (Humphrey 1955), on immune glomerulonephritis (Cochrane *et al.* 1965) and on vasculitis (Ward & Cochrane 1965). To facilitate studies on the role of PMNs in various conditions in the rat, we prepared an ANS with great similarities to that used by Simpson and Ross (1971; 1972) in the guinea-pig. As this has not previously been characterized and as, in spite of the studies of Simpson and Ross (1971), the kinetics of the PMN-depletion process has not been fully described, the purpose of the present study was to elucidate these points. A further aim was to determine whether the antiserum-induced neutropenia was followed by alterations in two of the cascade systems of the body, namely the complement system and the fibrinolytic system.

Materials and methods

Animals. Sprague–Dawley rats from the Alab farm, Vallentuna, Sweden, weighing 190–250 g, were used. The animals were fed with Ewos rat pellets and had free access to tap water up to the time of experimentation. Injections and blood sampling were performed under ether anaesthesia.

Preparation and characterization of ANS. ANS was prepared essentially as described by Simpson and Ross (1971; 1972). Rat PMNLs were harvested from peritoneal exudate 12 h after an intraperitoneal (i.p.) injection of 30 ml freshly prepared sterile filtered 3% proteose peptone (Oxoid Ltd., Basingstoke, Hants, UK). Following isolation of PMNs by Ficoll density gradient centrifugation at room temperature (Ficoll-Paque, Pharmacia, Uppsala, Sweden), the cells were briefly suspended in distilled water to haemolyze any red blood cells (RBCs) present, then washed twice in saline, counted and resuspended in saline to a concentration of 1×10^8 cells/ml. The final cell suspension consisted of >98% PMNs. Viability estimated by trypan blue exclusion was 97%. Three male adult New Zealand albino rabbits weighing 3.5–4.5 kg were then immunized with 50×10^6 PMNs diluted to 0.5 ml in an equal volume of Freund's complete adjuvant by intramuscular injection at each hind leg. The immunization and booster schedules were as described by Weir (1978). The animals were boostered every eighth week with the same amount of cells in Freund's incomplete adjuvant. Twice between each booster dose blood was collected from the central ear artery and allowed to coagulate, and after centrifugation the sera from the different rabbits were pooled, heat inactivated at 56°C for 30 min and filtered through a double Millipore filter (pore sizes 0.45 and 0.22 μm).

Control serum. Normal rabbit serum (NRS) was collected from each of the three rabbits before immunization and processed in the same manner as ANS.

Absorption of ANS. Haemagglutinins were eliminated by absorption to 0.1 ml of packed RBCs/ml ANS. Anti-serum antibodies were removed by absorption with 0.05 ml rat

serum/ml ANS, and anti-lymphocyte antibodies by absorption against lymphocytes harvested from the rat spleen and thymus (twice, 8×10^6 lymphocytes/ml ANS). All absorptions were carried out for 1 h at room temperature under shaking, whereafter the samples were centrifuged. The absorbed serum was precipitated three times with 70% ammonium sulphate resuspended to the initial volume and thereafter dialysed repeatedly against saline.

Titration of ANS. Leucoagglutination was essentially performed according to the method of Steinberg and Martin (1945) and titres against PMNs, RBCs and lymphocytes were determined. Indirect immunofluorescence titration of ANS was performed by a modification of the 'chess bord titration method' (Karlsson & Thal 1974). Briefly, 1×10^6 PMNs or 2.2×10^6 platelets per tube were mixed with absorbed ANS in dilutions, incubated for 30 min at 4°C and washed twice, whereafter FITC-labelled swine-anti-rabbit globulin (DAKO Labs., Denmark) was added in two-fold dilutions. Samples were examined in reflected light and the highest dilution of ANS with distinct fluorescence was considered to be the plateau titre of the serum.

Organ distribution of ^{51}Cr-labelled PMNs and effects on total complement (CH_{50}). Animals were injected with 400 µl of ^{51}Cr-labelled PMNs (16×10^6 PMNs; $2.8–3.1 \times 10^6$ ct/min) i.v. One hour later about 1 ml of blood was withdrawn for preparation of plasma and serum, measurement of the hematocrit, and cell count, whereafter 21 mg/kg b.w. of ANS or NRS was injected i.v. Five or 120 min later the rats were bled by aortic puncture for determination of the haematocrit and radioactivity in plasma and organs, and of complement activity in serum. Three minutes before the animal was killed, 5 µCi of ^{125}I-human serum albumin was injected i.v. as a plasma tracer. Accumulation of ^{51}Cr activity in different organs was corrected for the contribution of blood-borne activity. The free radioactivity in plasma was calculated as:

$$^{51}\text{Cr released (\%)} = \frac{^{51}\text{Cr plasma activity} \times \left(1 - \frac{\text{Hct}}{100}\right)}{^{51}\text{Cr whole blood activity}}$$

The serum obtained for complement assay was stored in polypropylene tubes at −70°C until analysed.

Various methods. ^{51}Cr-labelling of peritoneal PMNs and radioactivity determinations in organ, blood and plasma samples were performed as described by Lundberg and Arfors (1983).

Protein was determined by the Biuret method. Plasminogen activators and the fibrinogen concentration in plasma were measured according to the methods of Nilsson and Olow (1962). The plasminogen concentration, antiplasmin activity and α_2-anti-plasmin immunoreactive material in plasma were determined as described by Högstorp *et al.* (1981). CH_{50} in serum was assayed according to the method of Nilsson and Nilsson (1984).

Statistics. Differences between groups were evaluated by Student's *t*-test for unpaired or paired variables. All values given in the following are mean ± s.d.

Experiments and results

In-vitro characterization of ANS

The unabsorbed and unprecipitated serum had a protein concentration of 65–77 g/l and the absorption and precipitation procedure reduced the concentration to 7–9 g/l. Unabsorbed ANS had a leucoagglutinin titre against rat neutrophils of 1/5120 (Table 1). After absorption the titre diminished one step to 1/2560. Before absorption the titre against lymphocytes was 1/320, but absorption did not decrease this by more than one

Table 1. In-vitro characterization of ANS

	Agglutination titres			Chess board titres	
	PMNs	RBCs	Lymphocytes	PMNs	Platelets
Preimmune serum	<1/10	<1/10	<1/10	1/20	nd
Unabsorbed ANS	1/5120	1/20	1/320	nd	nd
Absorbed ANS	1/2560	<1/10	1/160	1/2560	1/20

nd Not determined.

step to 1/160. There was only borderline reactivity to RBCs before absorption, and none after absorption. Pre-immune serum obtained from rabbits (NRS) had no agglutination titres to any of the tested components.

Chess board titration by indirect immunofluorescence revealed a plateau titre against PMNs for the absorbed serum of 1/2560 and for NRS of 1/40. There was a very low titre against platelets (1/20).

Intravenous administration of ANS

Effect of the number of peripheral cells. Administration of ANS in three different doses, 10.5, 21 and 42 mg/kg body weight (b.w), corresponding to 0.3, 0.6 and 1.2 ml respectively, resulted in greatly varying effects on the number of peripheral cells (Figs 1 & 2). The highest dose was followed by a profound decrease in the number of circulating PMNs to a minimum value 5 min after the injection in all three animals which received this dose. After injection of 10.5 and 21 mg/kg b.w., i.e. 0.3 and 0.6 ml, four out of six animals exhibited a similar temporary neutropenia. Administration of ANS (all doses) was also followed by a simultaneous decrease in the number of circulating platelets, which, however, reached a minimum as early as 2.5 min after the injection. Injection of NRS had no effect on the number of peripheral PMNs or platelets. The large differences in the number of peripheral PMNs in the later part of the observation period after administration of ANS was partly attributed to different degrees of stress between the animals due to repeated ether anaesthesia.

In animals injected with ^{51}Cr-labelled PMNs and killed 5 min after an i.v. injection of ANS 21 mg/kg b.w. there was an increased radioactivity in the lungs and spleen and a simultaneous increase in free radioactivity in the plasma (Fig. 3). Two hours later there was no difference in the radioactivity in the liver, spleen, kidney or skeletal muscle between animals given ANS and NRS, but the amount of radioactivity in the lung was considerably lower and the concentration of free radioactivity in the plasma higher in ANS-treated animals (Fig. 3).

Intravenous injection of ANS was followed by a decrease in total complement (CH_{50}) to $1.7 \pm 0.4\%$ of the baseline value at 5 min, while in NRS-treated animals the complement activity was unchanged. Two hours later about 50% of the complement activity was restored and CH_{50} was now $53 \pm 10\%$ of baseline value (Fig. 3).

Intraperitoneal administration of ANS

Twelve hours after i.p. injection of ANS in different doses (3.5, 10.5 and 21 mg/kg b.w., i.e. 0.1, 0.3 and 0.6 ml) there was a profound decrease in the number of circulating PMNs (Fig. 4, Table 2). Three hours after i.p. injection a substantial reduction in the number of PMNs was observed only after administration of the higher doses, 10.5 and 21 mg/kg. Only the highest dose, 21 mg/kg

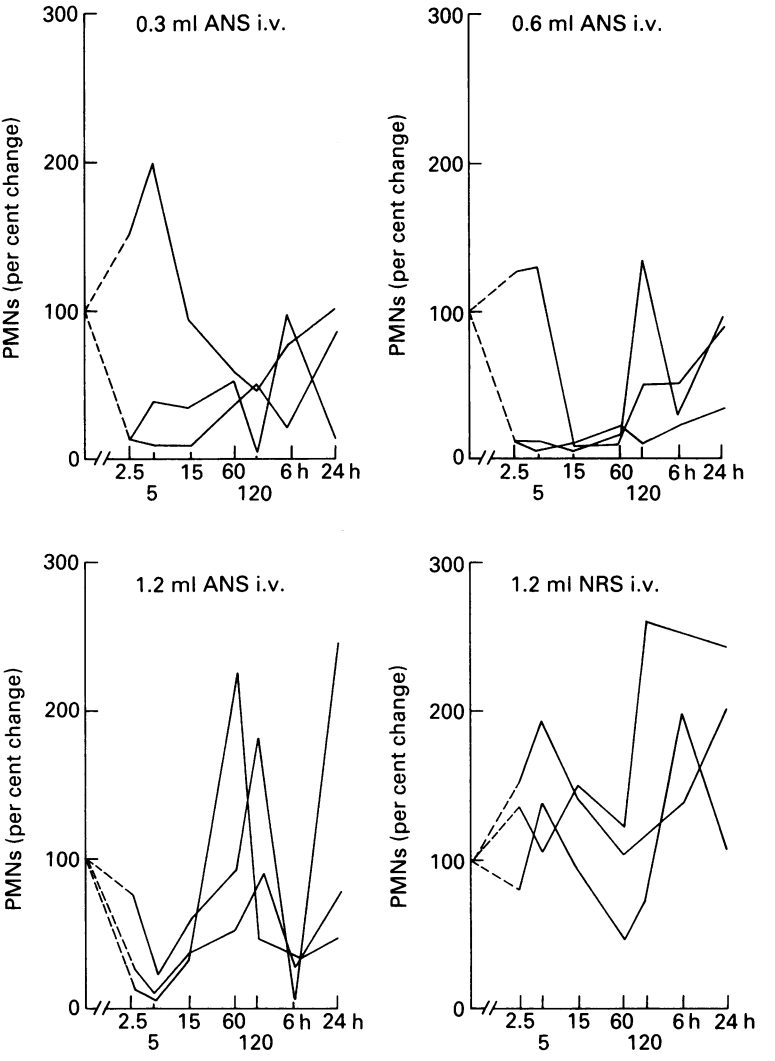

Fig. 1. Changes in the number of PMNs in individual rats after different single i.v. doses antineutrophil serum (ANS) and normal rabbit serum (NRS) 1.2 ml. Blood was withdrawn before and 2.5, 5, 15, 60, 90, 120 min, 6 h and 24 h after injection.

b.w., was followed by a prolonged profound neutropenia that lasted at least 6 h. Twenty-four hours after injection of ANS, the PMNL count, regardless of the dose given, was still below the baseline value. As seen from Table 2, the total leucocyte count was significantly reduced after induction of neutropenia, but there were no significant changes in the number of platelets. NRS, 21 mg/kg b.w., had no effect on the total leucocyte, PMN or platelet count.

Fibrinolysis parameters and complement. In the studies on the effect of fibrinolysis parameters, ANS in a dosage of 21 mg/kg b.w. was given i.p. and one or more additional doses of 10.5 mg/kg b.w. were given i.p. at 24 h or later in order to prolong the neutro-

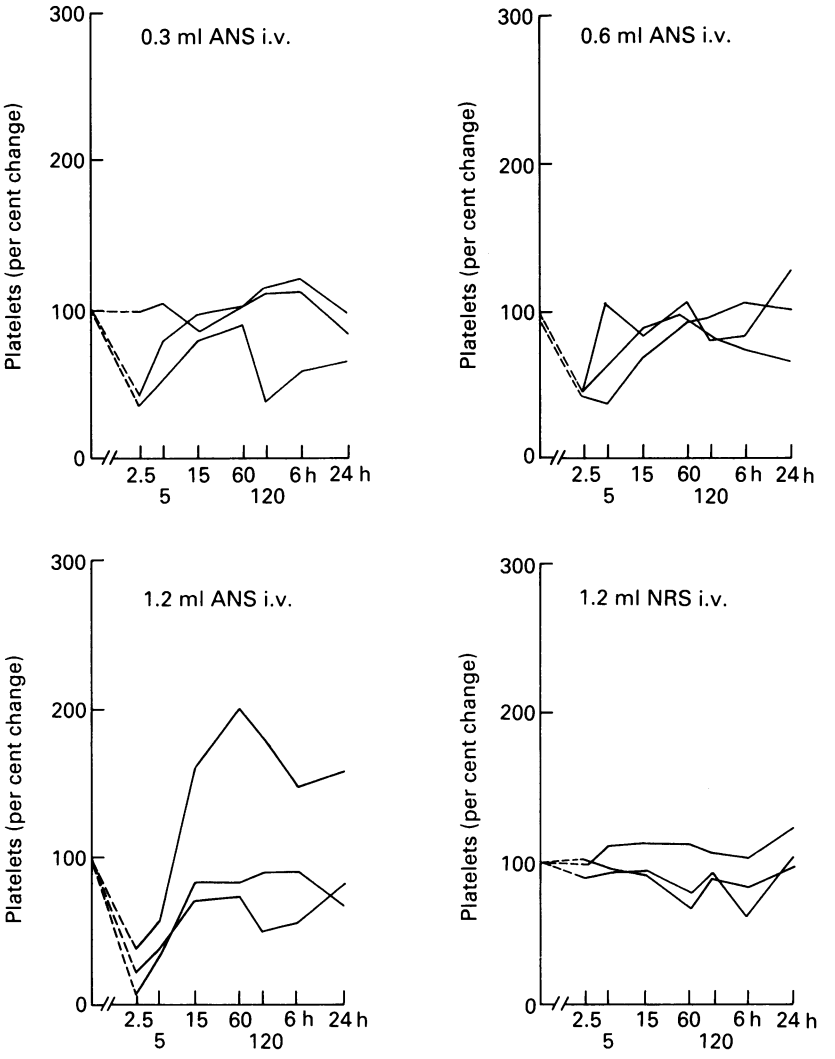

Fig. 2. Changes in the platelet count in individual rats after different single i.v. doses antineutrophil serum (ANS) and normal rabbit serum (NRS) 1.2 ml. Blood was withdrawn before and 2.5, 5, 15, 60, 90, 120 min, 6 h and 24 h after injection.

penia to 48 h. As seen in Table 2, the total leucocyte count was decreased to about half, while the number of PMNs decreased to zero. ANS had no significant effect on the number of platelets, on the fibrinogen concentration in plasma, on α_2-antiplasmin activity, α_2-antiplasmin immunoreactive material or on the plasminogen concentration. At 48 h the plasminogen activator concentration showed a slight but non-significant increase. The variation between the values for this parameter probably reflects the various degrees of stress caused by the repeated anaesthesia.

Twelve hours after i.p. administration of ANS in a dosage of 21 mg/kg b.w. CH_{50} was unchanged (Fig. 3).

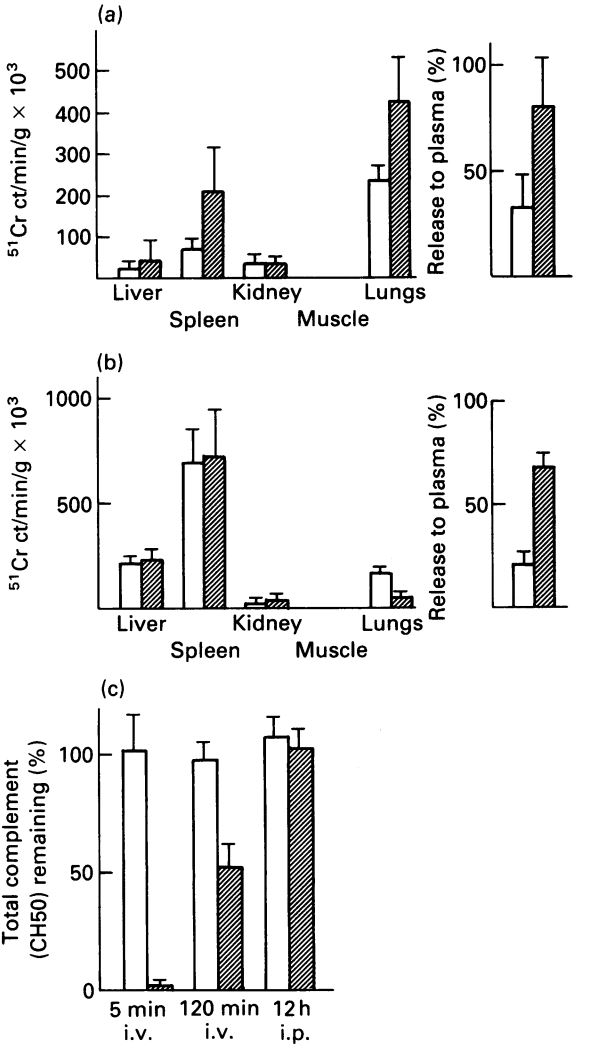

Fig. 3. Effect of 21 mg/kg b.w. of antineutrophil serum (ANS,▨) and normal rabbit serum (NRS,□) given i.v. on the distribution of ^{51}Cr-radioactivity to different organs on the percentage of activity released to plasma after 5 min (*a*) and 120 min (*b*). Alterations in total complement (CH_{50}) 5 min and 120 min after i.v. injection of 21 mg/kg b.w. ANS and NRS and at 12 h after the same dose i.p. (*c*). $n=6$ in each group.

Discussion

The rabbit antiserum raised against rat PMNLs had good specificity against those cells, with an anti-PMNL titre 16 times higher than the titre against rat lymphocytes. After i.v. injection there was a very transient neutropenia, thrombocytopenia and deposition of ^{51}Cr PMNs in the lungs, followed by release of ^{51}Cr radioactivity to plasma and a consumption of complement. After i.p. injection there was an isolated neutropenia and no impact on the fibrinolytic or complement system.

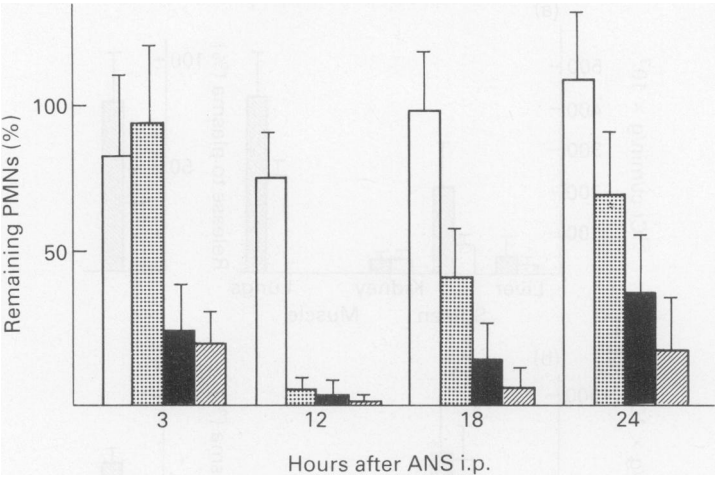

Fig. 4. Percentage numbers of PMNLs remaining at various times after i.p. injection of normal rabbit serum (NRS) and of various doses of antineutrophil serum (ANS). $n=7$ in each group. □, NRS 21 mg/kg; ▨, ANS 3.5 mg/kg; ■, ANS 10.5 mg/kg; ▨, ANS 21 mg/kg.

Table 2. Effect of antineutrophil serum (ANS) on leucocytes and platelets and on some fibrinolysis parameters ($n=6$). Twenty-one mg/kg b.w. ANS was given i.p. at zero time. Additional dose(s) of 10.5 mg/kg b.w. ANS was given i.p. if needed to maintain neutropenia

	Before	12 h	24 h	48 h
Leukocytes total (10^9/l)	15.6±2.0	6.6±2.0*	7.3±1.0*	8.4±1.2*
PMNLs (10^9/l)	1.0±0.3	0	0	0
Mononuclear cells (10^9/l)	14.7±1.7	6.6±2.0	7.3±1.0	8.4±1.2
Platelets (10^9/l)	980±187	845±160	875±175	820±102
Fibrinogen (g/l)	2.08±0.29	2.34±0.22	2.44±0.37	2.57±0.40
α_2-PI activity (%)	100±14	94±11	—	96±7
α_2-PI immunoreactive material (%)	100±5	97±4	—	109±6
Plasminogen (%)	100±5	92±15	105±2.0	105±29
Plasminogen activators, mm²	312±64	271±46	—	472±196

* $P<0.05$ versus 0 h.

Simpson and Ross (1971; 1972) noted coincident reductions of lymphocytes and PMNs after administration of their ANS. The same was observed in the present study and probably reflected the cross reactivity between PMNs and lymphocytes seen in the in-vitro assay. The absorption procedure which was carried out to increase the specificity resulted in a one step lower titre both against PMNs and against lymphocytes. From this it may be questioned whether the absorption was of any benefit.

The dramatic decrease in the complement activity and simultaneous accumulation of ^{51}Cr-labelled cells in the lungs after i.v. injection of ANS suggests that the initial phase of PMN elimination consists in complement-induced sequestration in the lungs. In fact, complement activation with cobra venom factor has been used by Till *et al.*

(1982) to obtain pulmonary sequestration of PMNs in the rat. The transient thrombocytopenia was supposed to be due to co-sequestration in the lung microvascular bed as i.v. injection of ANS in animals previously made neutropenic by i.p. injections did not have any impact on the peripheral platelet level (preliminary studies, data not presented). The increased release of ^{51}Cr activity to plasma even very shortly after an i.v. injection of ANS indicates that cytolytic processes occur simultaneously with the pulmonary sequestration of PMNs. Two hours after the i.v. injection of ANS, the ^{51}Cr uptake in the lungs was considerably lower than in NRS treated animals. This supports the previously established fact that the pulmonary vascular bed constitutes a considerable reservoir for PMNs (Bierman *et al.*, 1955).

The accumulation of ^{51}Cr in spleen and liver is also in accordance with the observation by Simpson and Ross (1971) of an early accumulation of PMNs in these organs. They noticed a phagocytosis of seemingly intact PMNs by macrophages.

In contrast to the undesired thrombocytopenia and complement activation and the only transient neutropenia observed after i.v. administration of ANS, an i.p. injection was followed by a more specific response with no effect on the complement or fibrinolytic system. After this mode of administration the neutropenia was considerably prolonged, indicating that ANS given intraperitoneally could be a valuable tool in studies on the pathogenetic role of PMNLs in various procedures. Although it also has a certain impact on the number of circulating mononuclear cells, this procedure seems to be the mildest way to induce neutropenia in the rat.

Acknowledgement

We are grateful to Dr Ulf Nilsson, The Blood Center, University Hospital, Uppsala, for helping us with the complement determinations. The technical assistance of Elisabeth Persson and Lars-Göran Axelsson is gratefully acknowledged. We also thank Ms Annie Lindbom for preparing the manuscript. This investigation was supported by Stenholms Foundation for Surgical Research.

References

BIERMAN H.R., KELLY K.H. & CORDES F.L. (1955) the sequestration and visceral circulation of leukocytes in man. *Ann. NY Acad. Sci.* **59**, 850–862.

COCHRANE C.G., UNANUE E.R. & DIXON F.J. (1965) A role of polymorphonuclear leukocytes and complement in nephrotoxic nephritis. *J. Exp. Med.* **122**, 99–116.

FLICK M.R., PEREL G. & STAUB N.C. (1981) Leukocytes are required for increased lung microvascular permeability after microembolism in sheep. *Cir. Res.* **48**, 344–351.

HUMPHREY J.H. (1955) The mechanism of Arthus reactions. II. The role of polymorphonuclear leukocytes and platelets in reverse passive reactions in the guinea-pig. *Br. J. exp. Path.* **36**, 283–289.

HÖGSTORP H., JACOBSSON H. & CARLIN G. (1981) Studies on α_2-anti-plasmin in the rat. *Thrombos. Res.* **21**, 247–253.

JOHNSON A. & MALIK A.B. (1980) Effect of granulocytopenia on extravascular lung water content after micro-embolization. *Am. Rev. Resp. Dis.* **122**, 561–566.

KARLSSON K.-A. & THAL E. (1974) Studies on the detection of salmonellae in fishmeal by the direct and indirect immunofluorescent method. *Nord. Vet. Med.* **26**, 492–498.

LAWRENCE J.S., CRADDOCK C.G. JR. & CAMPBELL T.N. (1967) Anti-neutrophilic serum, its use in studies on white blood cell dynamics. *J. Lab. Clin. Med.* **69**, 88–101.

LUNDBERG C. & ARFORS K.-E. (1983) Polymorphonuclear leukocyte accumulation in inflammatory dermal sites as measured by ^{51}Cr-labelled cells and myeloperoxidase. *Inflammation* **7**, 247–255.

NILSSON I.-M. & OLOW B. (1962) Determination of fibrinogen and fibrinolytic activity. *Thromb. Diath. Haemorrh. (Stuttg.)* **8**, 297–310.

NILSSON U.R. & NILSSON B. (1984) Simplified assays of hemolytic activity of the classical and alternative complement pathways. *J. Immunol. Methods* **72**, 49–59.

SHASBY D.M., FOX R.B., HARADA R.N. & REPINE J.E. (1982) Reduction of the edema of acute hyperoxic lung by granulocyte depletion. *J. Appl. Physiol.* **52**, 1237–1244.

SIMPSON D.M. & ROSS R. (1971) Effects of heterolo-

gous on antineutrophil serum in guinea pigs. *Am. J. Pathol.* **65**, 79–95.

SIMPSON D.M. & ROSS R. (1972) The neutrophilic leukocyte in wound repair. A study with antineutrophil serum. *J. Clin. Invest.* **51**, 2009–2023.

STEINBERG R. & MARTIN A. (1945) Factors influencing leucoagglutination by antileucocytic sera. *J. Immunol.* **51**, 421–426.

TAHAMONT M.V. & MALIK A.B. (1983) Granulocytes mediate the increase in pulmonary vascular permeability after thrombin embolism. *J. Appl. Physiol.: Respirat. Environ. Exercise Physiol.* **54**, 1489–1495.

TILL G.O., JOHNSON K.J., KUEKEL R. & WARD P. (1982) Intravascular activation of complement and acute lung injury. Dependency on neutrophils and toxic oxygen metabolites. *J. Clin. Invest.* **69**, 1126–1135.

WARD P.A. & COCHRANE C.G. (1965) Bound complement and immuno-logic injury of blood vessels. *J. Exp. Med.* **121**, 215–234.

WEDMORE C.V. & WILLIAMS T.J. (1981) Control of vascular permeability by polymorphonuclear leukocytes in inflammation. *Nature* **289**, 646–650.

WEIR D.M. (1978) *Handbook of Experimental Immunology.* Third edition. Oxford: Blackwell Scientific Publications. p. A3:12.